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Research Article

The role of Ynt1 in nitrate and nitrite transport in the yeast *Hansenula polymorpha*

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Abstract

Ynt1 is the only high-affinity nitrate uptake system in *Hansenula polymorpha.* Nitrate uptake was directly correlated with the Ynt1 levels and shown to be independent of nitrate reductase (NR) activity levels. Ynt1 failed to transport chlorate and, as a result, strains lacking *YNT1* were sensitive to chlorate, as is the wild-type. Nitrite uptake in a wild-type strain was partially inhibited by nitrate to levels shown by a *YNT1*-disrupted strain in which, in turn, nitrite transport was not inhibited by nitrate. It is concluded that nitrite uptake takes place by two different transport systems: Ynt1 and a nitrite-specific transporter(s). The nitrite-specific transport system was induced by nitrate; consistently, no induction was observed in strains lacking the transcription factor *YNA1*, which is involved in nitrate and nitrite induction of the nitrate assimilatory structural genes. Ynt1 presents its optimal rate for nitrite uptake at pH 6, while pH 4 was optimal for the specific nitrite uptake system(s). At pH 5.5, the contribution of Ynt1 to high-affinity nitrate and nitrite uptake was around 95% and 60%, respectively. The apparent K_m of Ynt1 for nitrate and nitrite is in the μM range, as is the specific nitrite uptake system for nitrite. The analysis of the effect of the reduced nitrogen sources on nitrate assimilation revealed that glutamine inactivates nitrate and nitrite transport, dependent on Ynt1, but not the nitrite-specific system.

Keywords: **nitrate; nitrite; transport; yeast; *Hansenula polymorpha***

Introduction

In the yeast *Hansenula polymorpha*, genes encoding nitrate transporter (*YNT1*), nitrate reductase (*YNR1*) and nitrite reductase (*YNI1*) are clustered. The same cluster also contains two $Zn(II)_2Cys_6$ transcriptional factors encoded by *YNA1* and *YNA2*, both essential for nitrate induction of *YNT1,*

YNR1 and *YNI1*. *YNA1* or *YNA2* disruptants are unable to grow in nitrate or nitrite (Avila *et al*., 1995, 1998, 2002; Brito *et al*., 1996; Pérez *et al*., 1997).

The nitrate transporter Ynt1 has 12 putative transmembrane domains (Pérez *et al*., 1997). It has been included in the NNP (nitrate–nitrite porter) family, involved in high-affinity nitrate and/or nitrite transport (Forde, 2000), a member of the major facilitator superfamily (Pao *et al*., 1998). Ynt1 shows similarity to NrtA (formerly designated CRNA) and NrtB from *Aspergillus nidulans* (Unkles *et al*., 1991, 2001) and high-affinity nitrate transporters from plants and algae (Daniel-Vedele *et al*., 1998).

Multiplicity of nitrate transporters is a common feature found in yeast, filamentous fungi, algae and plants. In *H. polymorpha*, low-affinity nitrate transport, unrelated to Ynt1, has been observed in mutants lacking *YNT1*. However, the gene(s) involved in this transport have not yet been cloned (Machín *et al*., 2000). Two high-affinity nitrate transporters have been found in *A. nidulans* (Unkles *et al*., 2001). In the alga *Chlamydomonas reinhardtii*, four systems for nitrate and nitrite transport have been characterized thus far. These systems present differential specificity for nitrate and nitrite affinity and transcriptional regulation (Galván *et al*., 1996; Navarro *et al*., 2000; Quesada *et al*., 1994; Rexach *et al*., 1999). Genes encoding high- and low-affinity nitrate transporters have been isolated in higher plants (Crawford and Glass; 1998; Daniel-Vedele *et al*., 1998; Forde, 2000; Galván and Fernández; 2001).

The capacity of high-affinity nitrate transporters to transport nitrite and chlorite in fungi has been subject to controversy. Using *Xenopus* oocytes as a heterologous expression system, it was shown by electrophysiological methods that *A. nidulans* NrtA was able to transport nitrate, nitrite and chlorite (Zhou *et al*., 2000). However, a mutant lacking both NrtA and NrtB nitrate transporters does not show any decrease in growth on nitrite, highlighting the limited participation, if any, of these transporters in nitrite transport (Unkles *et al*., 2001). Transport of chlorate was not detected using the *Xenopus* oocytes heterologous expression system (Zhou *et al*., 2000), whereas an *A. nidulans* mutant strain lacking NrtA showed resistance to chlorate (Unkles *et al*., 2001).

The posttranslational responses of non-preferred nitrogen source permeases, such as Gap1, to the presence of preferred nitrogen sources, such as an ammonium compound or glutamine, have been widely documented in *Saccharomyces cerevisiae*, although they are not completely understood (Soetens *et al*., 2001; Springael *et al*., 1999; Springael and Andre, 1998). However, the response of a nitrate transporter to the presence of preferred nitrogen sources remains largely unexplored in yeast, filamentous fungi and plants.

In this work, the role of Ynt1 in nitrate, nitrite, chlorate and chlorite transport, its contribution to cell nitrate supply, and the response to the presence of reduced nitrogen are analysed. It is shown that Ynt1, a high-affinity nitrate transporter in fungi, is also able to transport nitrite with high affinity. The presence of glutamine in the medium triggers the inactivation of the nitrate and nitrite transport through Ynt1. Moreover, we show that at least one high-affinity nitrite-specific transporter, inducible by nitrate and not inactivated by glutamine, is present in *H. polymorpha.*

Materials and methods

Yeast strains

These are listed in Table 1.

Growth conditions

Yeast were grown in liquid medium containing 0.17% yeast nitrogen base without ammonium sulphate and amino acids (YNB). The carbon source is indicated in each case. Ammonium chloride, sodium nitrate or sodium nitrite were commonly

Table 1. Yeast strains

Strain	Genotype	Source
NCYC 495	*leu1-1*, *ura3*	Gröningen
NCYC495 (WT)*	*LEU2*, *URA3*	La Laguna
MDP100	Δ*ynt1*::*URA3*; P_{MOX1}::pET1 (P_{MOX1}-*YNT1*-T_{AMO1})	La Laguna
MDP 1995	Δ*ynr1*::*URA3*	Avila *et al.* (1996)
NB 1996	Δ*yni1*::*URA3*	Brito *et al.* (1998)
NB 1997	Δ*ynt1*::*URA3*	Pérez *et al.* (1997)
NB 1998	Δ*yna1*::*URA3*	Avila *et al.* (1998)

* This strain was obtained by transforming the NCYC495 *leu1.1*, *ura3* with plasmids linearized at the *LEU2* and *URA3* genes.

used as nitrogen sources. Cell growth was followed at OD_{660}. Cell density is expressed as mg(wet weight)/ml.

Fusion of *YNT1* to *MOX1* promoter

The *H. polymorpha* expression plasmid pET1 (Tan *et al*., 1995) was used to fuse the *YNT1* ORF to the *MOX1* gene promoter to construct plasmid pMDP16. This plasmid was linearized at the *Stu*I site in the *MOX1* promoter to target its integration to the *MOX1* locus of the Δ*ynt1::URA3* strain to obtain MDP100 (Δ*ynt1::URA3, MOX1p-YNT1*). Most recombinant DNA procedures were performed according to Sambrook *et al*. (1989).

Nitrate and nitrite uptake rate assays

Nitrate or nitrite transport was inferred from extracellular nitrate or nitrite depletion. Nitrate or nitrite uptake rate experiments were carried out at the cell density indicated (1–50 mg/ml) at 37 °C in 0.17% YNB plus 2% glucose and the indicated concentrations of nitrate or nitrite. Modifications made to the media are always indicated. The uptake assay was initiated by the addition of either nitrate or nitrite to the cell suspension. At short time intervals (1–2 min), samples (usually 200 µl) were withdrawn and the uptake activity stopped with 0.35 M HCl. Once the assay was finished, samples were neutralized with 1 M Tris and centrifuged. Extracellular nitrate was determined colorimetrically as nitrite, according to Snell and Snell (1948). Nitrate was enzymatically transformed into nitrite. The enzymatic reaction was carried out in a 96-well microtitre plate at 30 °C for 1 h in a 60 µl reaction mixture containing: 50 mM Tris–HCl, pH 7.5; 2.25×10^{-3} U *Aspergillus* sp. NR (Roche); 75 µM NADPH; and samples with no more than 20 nmol nitrate. The enzymatic nitrate determination method was validated by high performance liquid chromatography (HPLC), which was carried out using an anionic exchange column (IC-pack; A. Waters) and 2.5 mM lithium hydroxide as the eluant. Nitrate and nitrite were detected with an UV detector at 214 nm.

To determine extracellular nitrate at low concentration, samples were concentrated by freeze-drying. The nitrate/nitrite uptake assay conditions were modified to avoid interference with the determination of nitrate, either enzymatically or by HPLC. Glucose and YNB were reduced to 0.2% and 0.017%, respectively, and cell concentration to 0.5–1 mg/ml. Samples were concentrated between 10- and 40-fold. In nitrite uptake assays, extracellular nitrite was determined directly according to Snell and Snell (1948).

In nitrate and nitrite uptake experiments the medium was buffered at different pH values: pH 3, 4 and 5 with 20 mM tartaric acid–Tris; pH 5.5 and 6 with 20 mM MES–Tris; and pH 7 and 8 with 50 mM Tris–MES. To determine whether any of the buffer solution components interfered with uptake assays, overlapping pH buffers using different components were used in nitrate and nitrite uptake experiments. No significant variations in nitrate and nitrite uptake were observed with regard to the buffer components used (data not shown).

Production of a polyclonal antiserum against Ynt1

The region of Ynt1 comprising part of the central hydrophilic loop and the seventh transmembrane span (residues 224–367) was used as an antigen. It was overexpressed in *E. coli* using the pRSETa plasmid (Invitrogen Corporation, San Diego, USA). The antiserum anti-Ynt1 was obtained from a New Zealand rabbit according to Harlow and David (1988).

Extraction of membrane proteins

50 mg cells were resuspended in 250 µl 50 mM Tris–HCl, pH 7.4, plus protease inhibitor cocktail (Roche). The cell suspension, containing glass beads (0.5 mm diameter), was vortexed five times for 1 min, keeping it on ice for 1 min between each vortex. An additional 250 µl resuspension buffer was added, and the sample was vortexed for 10 s. The glass beads and cell debris were discarded by two consecutive centrifugations (3200 rpm, 1 min) in a microfuge. The resulting supernatant was centrifuged at 15 000 rpm for 30 min at 4 °C, and the pellet obtained (particulate fraction) was resuspended in 70 µl Triton X-100 0.25 mg/ml. Protein concentration was determined according to Bradford (1977), taking into account Triton X-100 interference.

SDS–PAGE and Ynt1 immunodetection

20 µl of 4× sample buffer (12% SDS, 6% mercaptoethanol, 30% glycerol, 0.05% Serva blue G, 150 mM Tris–HCl, pH 7; Schägger; 1994) was added to 60 µl particulate fraction resuspended in Triton X-100. Samples were heated to 40 °C for 30 min, 20 µg protein per lane was loaded on 12% acrylmide gels. Proteins were transferred to a PVDF membrane (Hybond-P, Amersham Ibérica SA, Spain). Ynt1 immunodetection was carried out using the anti-Ynt1 antiserum (1/1500 dilution), and a goat anti-rabbit IgG antiserum (1/30 000 dilution) conjugated to peroxidase was used as a secondary antibody (Roche). Ynt1 was detected on the blot using the ECL chemiluminescent system (Amersham) according to the manufacturer's instructions.

Electron microscopy

Whole cells were fixed and prepared for immunocytochemistry as described previously (Waterham *et al.*, 1994). Immunolabelling was performed on ultrathin sections of unicryl-embedded cells, using antiserum against Ynt1 and gold-conjugated goat-anti-rabbit (GAR-gold) antibodies, following the directions of the manufacturer (Amersham, Arlington Heights, IL).

Other methods

NR activity was determined as described by González and Siverio (1992).

Results

Role of Ynt1 in nitrate uptake

To evaluate the contribution of Ynt1 to the total nitrate consumption of *H. polymorpha* cells, nitrate uptake experiments were carried out using wild-type (WT) and Δ*ynt1::URA3* mutant strains. Under the experimental conditions used, nitrate uptake was directly proportional to the number of WT cells present in the assay medium (Figure 1). In contrast, no nitrate uptake was observed with Δ*ynt1::URA3* mutants. Nitrate uptake rate in the WT was approximately 0.5 nmol/min/mg cells. The fact that no nitrate uptake was observed in the *ynt1::URA3* mutant led to the conclusion that at low nitrate concentration, nitrate uptake is mainly attributed to Ynt1. Therefore Ynt1 is the only high-affinity nitrate transporter in *H. polymorpha*. However, this strain is still able to grow in nitrate, although at a much lower growth rate than the WT. Moreover, low-affinity nitrate uptake has been detected in a Δ*ynt1::URA3* strain (Machín *et al.*, 2000).

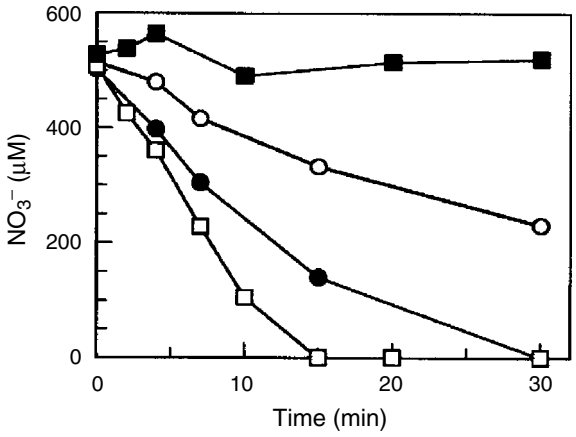

Figure 1. Nitrate uptake in WT and Δ*ynt1::URA3* strains. Cells grown in 5 mM ammonium were resuspended at 5 mg/ml in 0.5 mM nitrate for 2 h. The cells were then washed and resuspended in YNB plus 2% glucose; WT at 10 mg/ml (○), 30 mg/ml (●) and at 50 mg/ml (□); and the Δ*ynt1::URA3* mutant at 50 mg/ml (■). Nitrate uptake assays were triggered with 0.5 mM nitrate, samples for measurement of extracellular nitrate were taken at the appropriate times. The experiments were repeated three times without significant differences; the results shown are from a single experiment

To ascertain whether Ynt1 is the rate-limiting step in the nitrate assimilation pathway, the relationship between nitrate uptake and Ynt1 levels was determined. To address this question, the MDP100 strain was used. In this strain *YNT1* transcription is under the control of the *MOX1* promoter, which is induced by methanol and repressed by glucose (Gödecke *et al.*, 1994). Ynt1 usually localises in the plasma membrane. It was thought that overexpression of Ynt1 from the *MOX1* promoter could result in erroneous localization of Ynt1 to other cellular compartments. This would lead to misinterpretation of results. To exclude this possibility, Ynt1 was immuno-located by electron microscopy in MDP100 cells. As shown in Figure 2, Ynt1 is located exclusively in the plasma membrane.

To induce *YNT1* independently from the rest of the nitrate assimilatory genes, MDP100 cells were

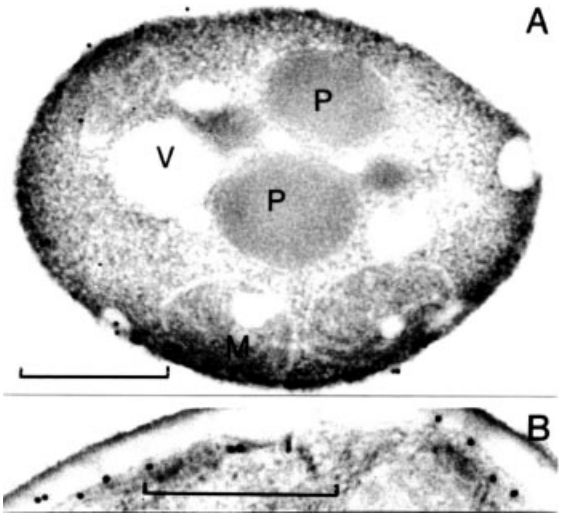

Figure 2. Membrane location of Ynt1 in a strain expressing *YNT1* under the *MOX1* promoter. (A) Survey of methanol-grown MDP100 cells overexpressing Ynt1, labelled with anti-Ynt1/GAR-gold. Labelling is exclusively located at the cell membrane. The high magnification (B) shows the labelled cell membrane in closer detail. M, mitochondrion; P, peroxisome; V, vacuole. The marker represents 0.5 μm

incubated in methanol plus ammonium, following incubation in glycerol. To produce increasing amounts of Ynt1, MDP100 cells were incubated in methanol for increasing time intervals. Subsequently, repression of the *MOX1* promoter and induction of NR were carried out by transferring cells to 4% glucose plus 1 mM nitrate for 1 h. Nitrate uptake and NR activity were determined in each sample, as shown in Figure 3. Nitrate uptake was directly proportional to the increasing levels of Ynt1 expressed during methanol induction. Moreover, the variation of the NR activity did not affect the nitrate uptake rate. Therefore, nitrate uptake rate is directly proportional to the amount of Ynt1, and independent of NR levels. A further conclusion made from this experiment, is that extracellular nitrate uptake depletion assays are a good method for determining nitrate transport in *H. polymorpha.*

With regards to Ynt1 affinity for nitrate, no differences in the nitrate depletion rate assays were found using extracellular nitrate concentrations of 25–250 μM (data not shown). This suggests that the K_m of Ynt1 for nitrate could be lower than 25μM. The K_m determined by the Eadie–Hofstee plot was 2 μM nitrate, while the V_{max} was around 0.5 nmol NO_3/min/mg cells (Figure 4).

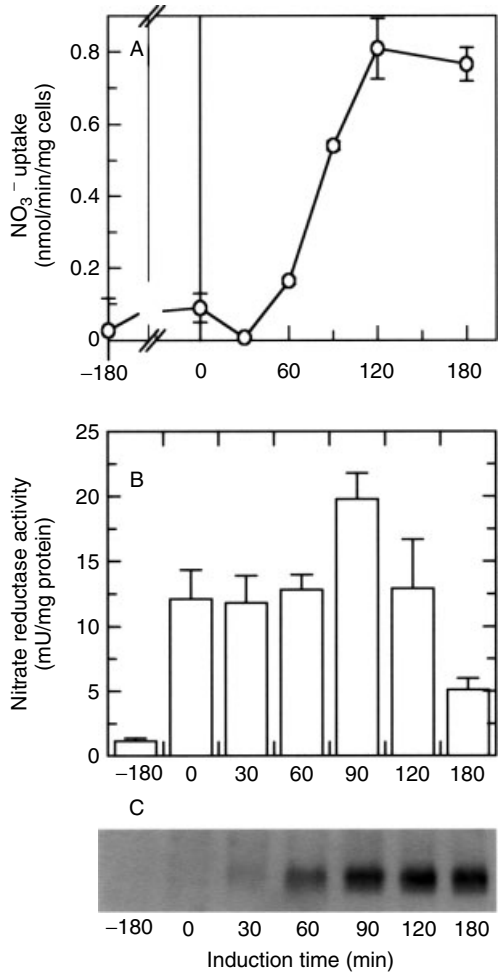

Figure 3. Correlation of Ynt1 levels with nitrate uptake rate. In MDP100 cells grown in glucose plus ammonium, *MOX1-YNT1* was derepressed in 0.5% glycerol plus 1 mM ammonium for 3 h, and then induced in 0.5% methanol plus 1 mM ammonium for the indicated times. Samples were harvested by centrifugation, incubated for 1 h in 4% glucose and 0.5 mM nitrate to repress *MOX-YNT1* and induce nitrate inducible genes, respectively. Subsequently, nitrate uptake (A), nitrate reductase activity (B) and Ynt1 levels (C), were determined for each sample. To measure Ynt1 levels, 10 μg of the particulate fraction was subjected to SDS-PAGE and immunoblotted using anti-Ynt antiserum. The experiments were repeated three times; each value is expressed as the mean ± SD

In *H. polymorpha* nitrite is transported by Ynt1 and a nitrite-specific transport system(s)

To study whether Ynt1 was able to transport nitrite, nitrite uptake experiments were carried out using Δ*ynr1::URA3* and Δ*ynt1::URA3* mutants

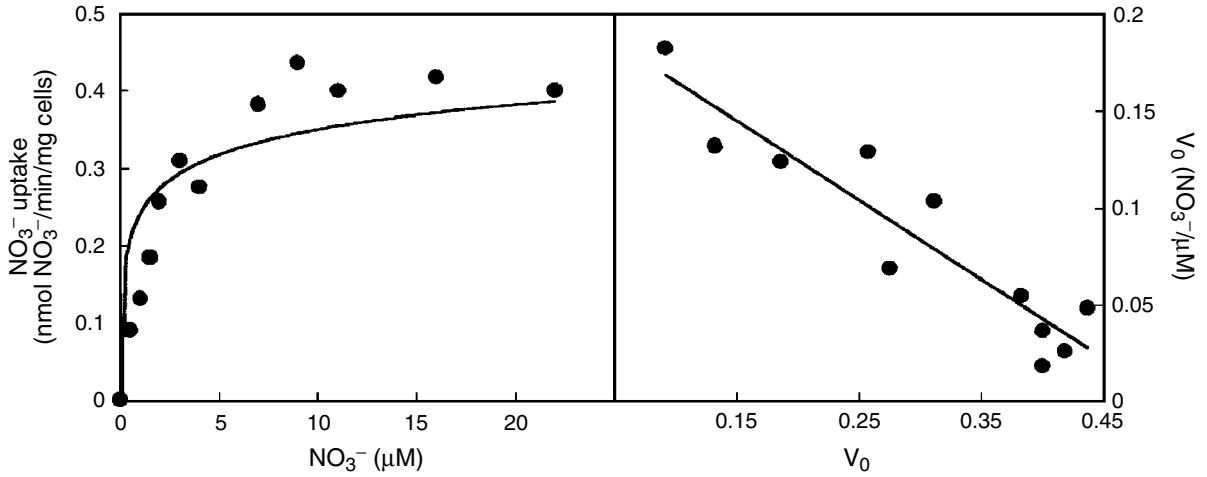

Figure 4. Kinetic characteristics of Ynt1 nitrate uptake. Wild-type cells grown in 5 mM ammonium were washed and resuspended at 5 mg/ml in 0.5 mM nitrate for 90 min. Cells were then resuspended in 0.2% glucose, 0.017% YNB, 20 mM MES-Tris, pH 5.5, at a cell concentration of 1-10 mg/ml, depending on the extracellular nitrate concentration used. Uptake experiments were triggered with nitrate. Extracellular nitrate depletion was followed for 21 min. Samples were taken every 3 min. The experiments were repeated three times; each value is expressed as the mean ± SD

(Figure 5). To avoid nitrite production and excretion (which could interfere with the nitrite uptake assay in the presence of nitrate) a Δ*ynr1::URA3* mutant was used, which expresses WT *YNT1* but lacks NR. The fact that nitrite uptake was three times higher in a Δ*ynr1::URA3* background than in a Δ*ynt1::URA3* background led us to conclude that Ynt1 also transports nitrite. However, since Δ*ynt1::URA3* mutants were shown to uptake nitrite, at least one additional transporter for nitrite is present in *H. polymorpha*.

Further evidence to corroborate the involvement of Ynt1 in nitrite transport was obtained by measuring the effect of nitrate on nitrite transport. The addition of 100 μM nitrate inhibited nitrite uptake in YNR1-deleted cells, to the levels of nitrite uptake observed in YNT1-deleted cells in the absence of nitrate (Figure 5). In addition, no inhibition of nitrite uptake by nitrate was observed in Δ*ynt1::URA3* mutants. This suggests specificity for nitrite of the Ynt1-independent nitrite uptake system (s). Our data allow us to conclude that Ynt1 transports nitrate and nitrite, and also strongly points towards the existence of a nitrite-specific uptake system (s).

With regard to the affinity of Ynt1 for nitrite, kinetic experiments showed that the K_m of Ynt1 for nitrite is in the μM range (Figure 6).

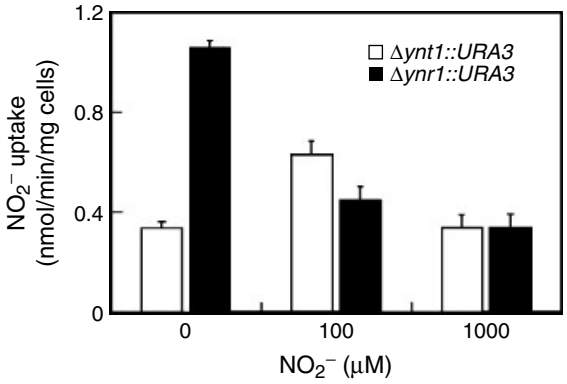

Figure 5. Ynt1 transports nitrite. Δ*ynt1::URA3* and Δ*ynr1::URA3* strains were grown in 5 mM ammonium, harvested, washed and resuspended for 2 h in 1 mM nitrate. To determine nitrite uptake, cells were resuspended at 10 mg/ml in a medium buffered with 10 mM MES-Tris, pH 5.5, in the absence and presence of 100 μM and 1000 μM nitrate. Nitrite uptake assays are triggered with 100 μM nitrite. Δ*ynr1::URA3* and Δ*ynt1::URA3* strains are represented with black and white bars, respectively. The experiments were repeated three times; each value is expressed as the mean ± SD

Characterization of nitrite transport

Figure 7 shows nitrite uptake rates measured at a pH range of 3–7 in WT and Δ*ynt1::URA3* cells. We found that at low pH (3–5), both strains reach

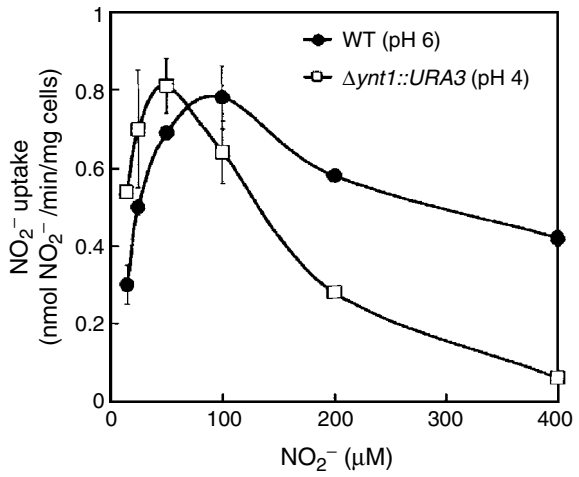

Figure 6. Nitrite uptake in WT and Δ*ynt1::URA3* strains at different extracellular nitrite concentrations. Cells grown in 5 mM ammonium were washed and resuspended in 1 mM nitrate, at 10 mg/ml, for 2 h. Cells were then resuspended at the appropriate cell density and nitrite uptake assays were triggered with 15, 25, 50, 100, 200 and 400 μM nitrite. The experiments were repeated three times; each value is expressed as the mean ± SD

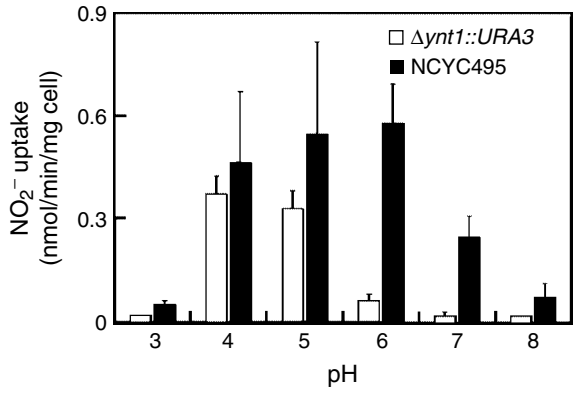

Figure 7. Effect of external pH on nitrite uptake in WT and Δ*ynt1::URA3* strains. Cells were grown in 5 mM ammonium, harvested, washed and resuspended in 1 mM nitrate for 2 h. To determine nitrite uptake, WT and Δ*ynt1::URA3* cells were resuspended at 10 mg/ml in media buffered to pH 3-8. Nitrite uptake assays were trigged with 100 μM nitrite. Δ*ynt1::URA3* and WT strains are represented with black and white bars, respectively. The experiments were repeated three times; each value is expressed as the mean ± SD

similar values of nitrite uptake, whereas at pH of around 6, only WT cells were able to transport nitrite efficiently. At pH 4, almost all nitrite transport occurs through the nitrite-specific system(s), since no important differences between WT and the Ynt1-null mutant cells were observed. However, at pH 6 practically all nitrite transport occurs through Ynt1. The lack of transport observed at pH 3 is most likely due to the uncoupling effect of nitrous acid. However, the incapacity of the nitrite-specific system to take up the protonated form of nitrite cannot be discarded. The optimal pH for the nitrite-specific system (s), pH 4, could also be explained if nitrite uptake at this pH was due to a simple diffusion of the nitrous acid, since nitrous acid presents a pKa of 3.37. However, this possibility is discarded by the fact that, at pH 4, proton ionophores such as 2,4-dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) strongly impair the nitrite uptake in Δ*ynt1::URA3* cells (Table 2), as expected for a proton symport system. Experiments designed to study the kinetic characteristics of the nitrite uptake system in Δ*ynt1::URA3* mutants showed that at pH 4, the optimal pH for nitrite uptake, the K_m for nitrite is in the μM range (Figure 6). Likewise, these experiments also showed that the increase of nitrite concentration to over 100 μM decreases nitrite uptake. This is inconsistent with simple diffusion of nitrite/nitrous acid into the cell. It is possible that at high nitrite concentrations the nitrous acid present at pH 4 could be at a concentration high enough to abolish nitrite transport due to its uncoupling effect.

To study the regulation of the nitrite uptake system(s) induction by nitrogen sources, Δ*ynt1::URA3* cells were grown in ammonium and transferred to media containing either nitrate or ammonium. As shown in Figure 8, nitrite depletion was only observed in cells previously incubated in nitrate-containing medium, unlike those cells incubated for 2 h in 5 mM ammonium. No nitrite uptake at pH

Table 2. Effect of proton ionophores on nitrite uptake in the Δ*ynt1::URA3* strain

	Control	CCCP	2,4 DNP
Δ*ynt1::URA 3*	100 ± 16	10 ± 6	7 ± 4

The cells were grown in 5 mM ammonium and resuspended at 5 mg/ml in 1 mM nitrate for 2 h. Nitrite uptake assays were performed at pH 4 plus 0.1 mM CCCP or 0.1 mM 2,4-DNP. A control assay was carried out without ionophores. The cells were incubated in these media for 5 min before adding nitrite. The results are expressed as percentage of nitrite uptake rate with respect to the control. The experiments were repeated three times; each value is expressed as the mean ±SD

4 was observed with *yna1* mutants, despite prior incubation in a nitrate-containing medium. This indicates that the induction of the nitrite uptake system (s) is also mediated by the $Zn(II)_2Cys_6$ transcription factor *YNA1*, which is involved in nitrate and nitrite induction of the nitrate assimilatory genes (Avila *et al*., 1998).

Effects of chlorate and chlorite on nitrate/nitrite uptake systems

Due to the importance of chlorate as a tool to study the nitrate assimilation pathway, we have determined whether chlorate/chlorite can be transported by any of the nitrate/nitrite transport systems in *H. polymorpha*.

To address the question of whether Ynt1 was able to transport chlorate, nitrate uptake was assayed at extracellular nitrate concentrations of 10 μM and 100 μM. Assays were performed in the presence of chlorate at the same concentration, or five-fold higher than the extracellular nitrate concentration used. Under these conditions nitrate uptake was not affected by chlorate (results not shown). Similar results were observed with WT and Δ*ynt1::URA3* mutant cells in the presence of chlorate. As expected, Δ*yna1::URA3* cells, in which the *YNR1* gene is not expressed, were much more resistant to chlorate (Figure 9). Furthermore, chlorate does not affect nitrite uptake at pH 4 in a Δ*ynt1::URA3* background (data not shown). This suggests that the nitrite-specific system does not transport chlorate.

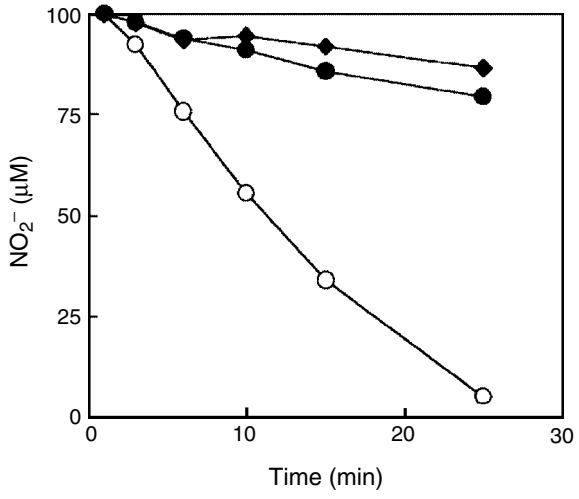

Figure 8. Nitrate induction and ammonium repression of the nitrite transport system. Δ*ynt1::URA3* and Δ*yna1::URA3* strains were grown in 5 mM ammonium. Cells were harvested, and resuspended at 10 mg/ml for 2 h; Δ*ynt1::URA3* cells in 1 mM nitrate (○), or 5 mM ammonium (●) and Δ*yna1::URA3* cells in 5 mM ammonium (♦). Cells were then harvested and resuspended at 5 mg/ml in YNB plus glucose, buffered at pH 4 with 20 mM tartaric acid-Tris; nitrite uptake assays were triggered with 100 μM nitrite. The experiments were repeated three times without significant differences. Results from a single experiment are shown

The effect of chlorite on Ynt1 nitrate/nitrite transport and on the nitrite-specific system was assayed. In addition, the effect of chlorite on the growth of WT cells, *ynt1* and *yna1* mutant cells cultured on nitrate-containing media over a range pH was measured. It was observed that all strains show a very similar sensitivity to chlorite, unlike chlorate, thus indicating that Ynt1, nitrite and NR are not involved in the sensitivity to chlorite. Furthermore, no differences between WT and Δ*ynt1::URA3* strains were found at either pH 4 or pH 6 (Figure 9). Chlorite inhibition assays carried out on Ynt1 nitrite/nitrate uptake at pH 6, and in the *YNT1*-null mutant strain at pH 4, showed a significant drop in nitrite uptake in both strains (data not shown). This indicates that both systems (Ynt1 and the nitrite-specific system) might transport chlorite. However, the non-specific toxic effect of chlorite on the cells, consistent with that observed in the growth experiments, casts doubt on the capacity of Ynt1 and the nitrite-specific system to transport chlorite.

Glutamine inactivates Ynt1 nitrate/nitrite uptake but not nitrite-specific uptake system(s)

To study further the characteristics of nitrate and nitrite transport in *H. polymorpha*, the postranslational response to the presence of reduced nitrogen sources was explored. We studied the effect of glutamine on cells grown in nitrate. Glutamine brought about a rapid inactivation of nitrate and nitrite uptake, and the degradation rate of Ynt1 increased notably (Figure 10A, B). Thus far, it is not clear whether the inactivation of the nitrate/nitrite uptake is a direct consequence of the proteolytic degradation of Ynt1, or whether covalent modifications of Ynt1 are also involved. To confirm that the inactivation of nitrate uptake is targeted to Ynt1, NR activity was measured during the experiment. No NR inactivation was observed (Figure 10A).

The effect of glutamine on the nitrite-specific system was also studied (Figure 10C). The Δ*ynt1::*

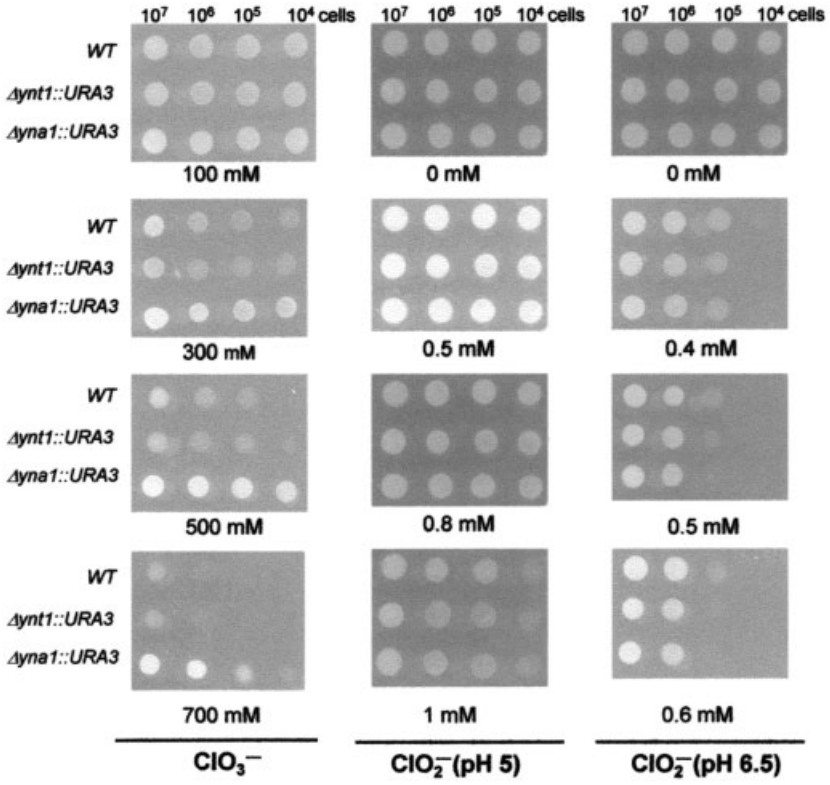

Figure 9. Effect of chlorate and chlorite on the growth of WT, Δ*ynt1::URA3* and Δ*yna1::URA3* strains. Cells grown in 5 mM ammonium were washed thoroughly and 10^7, 10^6, 10^5 and 10^4 cells were spotted onto solid medium containing 2% glucose, 0.17% YNB, 50 mM sodium nitrate, 0.2 mM urea and either chlorate or chlorite at the indicated concentrations. Chlorite-containing media were buffered at pH 5 with 20 mM tartaric acid-Tris and at pH 6.5 with 20 mM MES-Tris. The cells were grown for 3 days

URA3 mutant was grown in ammonium, and the nitrite-specific system was induced with nitrate. Glutamine did not inactivate the nitrite-specific system. This suggests that the nitrite-specific system is not post-translationally regulated. This is in contrast to the Ynt1 transporter, at least in the presence of glutamine.

Discussion

In this work the role of Ynt1 in nitrate and nitrite transport has been evaluated. Nitrate uptake was measured in WT and Δ*ynt1::URA3* strains. It was concluded that the uptake of nitrate takes place mainly through Ynt1. The dependence of nitrate uptake on Ynt1 and NR levels was also studied. As shown in Figure 2, nitrate uptake is dependent on the levels of Ynt1, and is independent of NR levels. Therefore, under the experimental conditions employed here, extracellular nitrate depletion can be considered as a measure of Ynt1-dependent nitrate transport. However, this method does not allow us to study nitrate transport for short time intervals. Using the developed method, a K_m for nitrate in the μM range was found for Ynt1. This allows us, based on kinetic data, to include Ynt1 in the high-affinity nitrate transporter group found in fungi, algae and plants (Forde, 2000).

A property of Ynt1 that also required investigation is its ability to transport nitrite, chlorate and chlorite. Two lines of evidence were considered, and it was concluded that Ynt1 transports nitrite. The first piece of evidence was the decrease of nitrite uptake in Δ*ynt1::URA3* cells when compared with the WT. The second is the inhibition of nitrite uptake to those levels observed in the Δ*ynt1::URA3* strain by nitrate. The reduction of nitrite uptake in the Δ*ynt1::URA3* strain could also

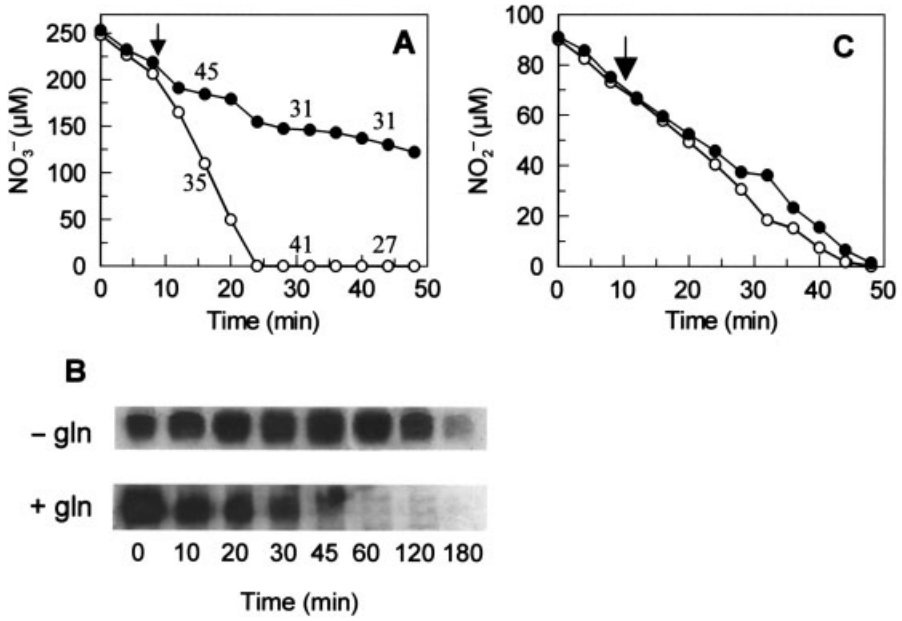

Figure 10. Effect of glutamine on nitrate and nitrite uptake in WT and Δ*ynt1::URA3* strains. (A) WT cells grown in 5 mM ammonium were resuspended at 5 mg/ml in YNB plus 2% glucose and 0.5 mM nitrate for 2 h, and were then subjected to nitrogen starvation for 1 h. The cells were then split into two aliquots and nitrate uptake was triggered with 0.25 mM nitrate. 10 min after nitrate uptake assay was initiated, 5 mM glutamine was added (↓) to one of the cultures. Nitrate depletion was followed in the presence (●) and absence (○) of glutamine. Nitrate reductase activity in nmol nitrite/min/mg protein was determined at 15, 30 and 45 min after starting the nitrate uptake assay. (B) Ynt1 levels in the presence and absence of glutamine. 10 μg protein from the particulate fraction was subjected to SDS-PAGE and immunoblot. (C) Δ*ynt1::URA3* cells were treated as in (A), except that the cell suspension was buffered at pH 5 and nitrite uptake was determined. The assay was triggered with 100 μm nitrite. The experiments were repeated three times without significant differences. Results from a single experiment are shown

be explained if the Ynt1-independent nitrite transporter were inefficiently induced by nitrate in a *ynt1* mutant, as previously shown for NR (Pérez *et al*., 1997). However, no increase of nitrite consumption in the Δ*ynt1::URA3* mutant was observed when higher nitrate concentrations were used in the medium to induce equivalent levels of NR between this strain and the WT (results not shown). Therefore, our results strongly suggest that Ynt1 transports nitrite, in addition to nitrate. This is the case for *C. reinhardtii* nitrate/nitrite transporters: CrNRT2.1 and CrNRT2.2 (Galván *et al*., 1996). The capacity of Ynt1 to transport nitrate and nitrite is consistent with that shown for *A. nidulans* NrtA in *Xenopus* oocyte experiments (Zhou *et al*., 2000). However, it has been claimed that NrtA and NrtB do not transport nitrite because an *A. nidulans* mutant strain lacking NrtA and NrtB is able to grow as efficiently in nitrite as the WT (Unkles *et al*., 2001). This does not rule out a role for NrtA and NrtB in nitrite transport, as the ability of these transporters to transport nitrite could be substituted for by additional nitrite transporters.

The presence of nitrite uptake in the Δ*ynt1::URA3* strain leads us to conclude that at least one additional nitrite transport system is present in this yeast. In contrast to the observations made with the Ynt1 transporter, no inhibition of nitrite uptake by nitrate is observed with the Ynt1-independent transporter. Therefore, it seems likely that the Ynt1-independent transporter is specific for nitrite. At pH 4, nitrite uptake occurs mainly via the nitrite-specific transporter(s), whereas at pH 6 nitrite transport occurs mainly through Ynt1. Proton ionophores such as DNP and CCCP inhibit nitrite uptake in *ynt1* mutants. This highlights the proton symport nature of the nitrite-specific transporter(s) and discounts the hypothesis for simple diffusion of nitrous acid across the plasma membrane at pH 4. The nature of the transporter(s) involved in nitrite uptake is unknown. However, the gene(s) encoding this transport system have no significant homology

with *YNT1*, since attempts made to isolate genes with *YNT1* homology by either DNA hybridization or PCR were unsuccessful.

Chlorate has been widely used to obtain mutants of the nitrate assimilation pathway, mainly in NR, since this enzyme is able to reduce chlorate to the very toxic compound chlorite. It has been proposed, in at least in two cases, that chlorate is transported into the cell through nitrate transporters, first by CHL1, a low-affinity nitrate transporter from *A. thaliana* (Tsay *et al*., 1993), and second by NrtA from *A. nidulans* (Unkles *et al*., 1991, 2001). However, Ynt1 does not seem to be involved in chlorate transport. Two facts support this conclusion: first, chlorate does not inhibit nitrate uptake; and second, there are no differences in growth between WT and Δ*ynt1* mutants when chlorate is present in the growth medium. In contrast to the *H. polymorpha ynt1* mutant described in this study, an *A. nidulans* mutant lacking NrtA showed resistance to chlorate, thus suggesting the capacity of NrtA to transport chlorate (Unkles *et al*., 2001). However, expression of NrtA in *Xenopus* oocytes suggests that NrtA is involved in chlorite transport instead of chlorate (Zhou *et al*., 2000). As a result, we also decided to investigate the effects of chlorite on *H. polymorpha*. Our results show that chlorite, or any compound present as a contaminant, inhibited growth regardless of the strain examined (WT, Δ*ynt1::URA3* or Δ*yna1::URA3*) (Figure 9). Although nitrate and nitrite uptake are decreased in the presence of chlorite, no conclusions can be drawn.

The effects of glutamine on nitrate and nitrite uptake by Ynt1-dependent, and nitrite-specific transport systems were investigated. Our results clearly show a distinctive response of Ynt1-dependent uptake to reduced nitrogen sources. This system is inactivated by glutamine. However, the nitrite-specific system(s) is not. A possible explanation for this is that the nitrite system is only subject to transcriptional, and not post-translational, regulation, unlike the Ynt1-dependent system. With regard to the inactivation of nitrate and nitrite uptake by Ynt1, it could be argued that the observations could be due to the inactivation of NR. However, neither ammonium or glutamine inactivate NR (Navarro *et al*., 2003). Further support for inactivation of Ynt1, but not NR by glutamine, is the observation that a strain expressing *YNR1* under the control of the *MOX1* promoter shows NR activity, even in an ammonium-containing medium (Navarro *et al*., 2003). In conclusion, our results strongly suggest that Ynt1 undergoes inactivation in response to reduced nitrogen sources. Total inactivation of nitrate uptake by ammonium or glutamine has been reported in the yeasts *Sporobolomyces roseus* and *Rhodotorula glutinis*. In contrast, partial inactivation has been found in *Candida nitratophila* and *C. utilis*. However, the nitrate assimilation pathway component responsible for inactivation of nitrate uptake has not been investigated further in these yeasts (Hipkin, 1989).

The results presented here go some way to bridge the gaps in our understanding between the physiological studies and the molecular basis of nitrate and nitrite transport in yeast. However, among the elements still unknown are genes that encode components of the nitrite-specific uptake system and the low-affinity nitrate uptake system. The discovery and subsequent cloning and investigation of these genes will broaden our overall understanding of nitrate and nitrite transport in yeast. The importance of post-translational regulation of nitrate and nitrite transport in both yeast and plants remains unclear. *H. polymorpha* could play an important role as a model system for understanding these processes.

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